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The plasma levels of conglutinin are heritable in cattle and low levels predispose to infection

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SUMMARY

Conglutinin, like mannan-binding lectin (MBL) and CL-43, is a serum collectin involved in the innate immune defence system. In man, low serum MBL concentrations, resulting from mutations in the collagen region, are associated with a common opsonic defect. Plasma levels of conglutinin in cattle were assayed by rocket immunoelectrophoresis to examine whether they were genetically determined. Samples were collected from calves (309 bull-calves and 260 heifers with complex pedigree relationships). The number of respiratory infections from the 42nd to 336th day of life was recorded. The number of infections was found to be genetically determined (heritability: $h^2 = 0{\cdot}31 \pm 0{\cdot}07$). A wide concentration range of conglutinin was found in plasma ($< 1{\cdot}25$–35 μg/ml for females, geometric mean 8·1 μg/ml, and $< 1{\cdot}25$–47 μg/ml for males, geometric mean 15·5 μg/ml), and the concentration was found to be genetically determined (heritability, $h^2 = 0{\cdot}52 \pm 0{\cdot}07$). The analysis revealed a negative association between disease frequency and the conglutinin levels ($-0{\cdot}56 \pm 0{\cdot}18$ for female; $-0{\cdot}50 \pm 0{\cdot}18$ for male). Levels of conglutinin below the detection limit of the assay (1·25 μg/ml) were found in 2% of the animals. If these animals are assumed to be homozygous for a single recessive allele causing low concentrations a gene frequency of 0·15 could be calculated. These findings suggests that selection for resistance against infectious disease is possible in cattle and that the level of plasma conglutinin may be a helpful trait in such a breeding scheme.

INTRODUCTION

Conglutinin belongs to a group of proteins called collectins, characterized by C-terminal carbohydrate recognition domains (CRDs) joined to collagenous regions.[1–4] The collectins promote phagocytosis, thus mediating the elimination of microorganisms. Five collectins are known: lung surfactant protein A and D (SP-A and SP-D), mannan-binding lectin (MBL), conglutinin and collectin-43 (CL-43). SP-A and SP-D are produced in alveolar type II cells and Clara cells, but recently both have also been found to be present in cells lining the gastrointestinal tract.[5,6] MBL, conglutinin and CL-43 are serum proteins produced in the liver. Conglutinin and CL-43 are only found in cattle and some other herbivores, whereas MBL has been found in the chicken as well as in all mammals examined.[7]

Conglutinin was the first mammalian lectin to be discovered. It was described at the beginning of the century as a component of bovine serum capable of agglutinating complement-coated erythrocytes – a reaction called conglutination.[8] Later it was shown that conglutination is mediated by binding of conglutinin to the complement component iC3b covalently attached to the erythrocytes.[9–12] Conglutination was early recognized as being related to infection through the observation of a decrease in conglutinating activity during infection with *Mycobacterium bovis*.[13] Furthermore, a decrease in the conglutinating activity of ox serum was observed during other systemic infections such as pneumonia, as well as at parturition and in connection with spontaneous abortion.[14–16] This phenomenon could reflect consumption, increased catabolism, or a down-regulation of the synthesis of conglutinin. In contrast, the level of MBL has been reported to increase during the acute phase reaction.[17] Several regulatory elements that might be responsible for this drop in concentration have been described in the 5′-flanking region of the conglutinin gene.[18]

Conglutinin shows opsonizing activity *in vitro* towards *Salmonella typhimurium* and *Escherichia coli*.[19] This activity

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Abbreviations: SP-A, lung surfactant protein A; SP-D, lung surfactant protein D; CRD, carbohydrate recognition domain; MBL, mannan-binding lectin; CL-43, collectin-43; TBS, tris-buffered saline; TBS/Tw, TBS–Tween 20; TBS/E, TBS–emulphogene

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was found to be dependent on the presence of an intact complement system, and serum deficient in factor I could not support the reaction. Results of sequential incubations suggested that conglutinin binds to iC3b deposited on the bacterial surfaces, whereupon the conglutinin-coated bacteria interacted with macrophages via a collectin receptor.

So far conglutinin is the only collectin for which protective activity has been demonstrated in experimental infections *in vivo*. Friis-Christiansen *et al.*[19] found that subcutaneous injections of conglutinin increased the survival of mice subsequently injected intravenously with a highly virulent strain of *Salmonella typhimurium*. Conglutinin interacts with influenza A virus, as shown by its inhibition of virus agglutinating activity as well as by its inhibition of infection *in vitro*.[20–23]

In man low serum concentrations of MBL are associated with recurrent infections in infancy.[24–25] Low levels of MBL correlate with point mutations in three codons of the collagen region of MBL, leading to poor expression of MBL.[24–26] In this report we find that the plasma concentration of conglutinin in cattle is genetically determined, dependent on sex and breed, and that low levels of conglutinin predispose to infections.

MATERIALS AND METHODS

Buffers and reagents

Tris-buffered saline (TBS): 140 mM NaCl, 10 mM Tris, 7·5 mM NaN_3, pH 7·2. TBS/Tw: TBS containing 0·05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, Merck-Schuchardt, Darmstat, Germany). TBS/E: TBS containing 0·05% (v/v) Emulphogene (polyoxyethylene 10 tridecyl ether, P-2393, Sigma, St. Louis, MO). Agarose gel electrophoresis buffer: 73·2 mM Tris, 24·3 mM barbital, 0·5 mM sodium lactate, 5 mM ethylenediamine tetra-acetic acid (EDTA), 2 mM NaN_3, pH 8·6.

Rocket immunoelectrophoresis

Anti-conglutinin antiserum (produced as described in ref. 27) was incorporated in the agarose gel (Litex HSA, Litex Industry, Copenhagen, Denmark) at 15 μl/cm^2.[28] The electrophoresis was run at 2·5 V/cm for 18 hr. Conglutinin was purified as described in.[29] The protein concentration in this preparation was established by quantitative amino acid analysis. This preparation was used as standard in the rocket immunoassay at a concentration range of 20 μg/ml to 1·25 μg/ml on 1·5 mm thick 1% (w/v) agarose gels. All plasma samples were tested undiluted. Samples with conglutinin concentration above 20 μg/ml were reanalysed after dilution.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

Electrophoresis was performed on 4–20% (w/v) polyacrylamide gradient gels with discontinuous buffers. Samples were reduced by heating at 100° for 3 min in 40 mM dithiothreitol, 1·5% (w/v) SDS, 5% (v/v) glycerol, 0·1 M Tris, pH 8·0, and carboxyamidated by the addition of iodoacetamide to 90 mM. Protein bands were detected by Coomassie Brilliant Blue.

For Western blotting the separated proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon P, Millipore, Bedford, MA).

The membrane was incubated with primary antibody (polyclonal rabbit anti-conglutinin antiserum diluted 1/10 000) and secondary alkaline-phosphatase-coupled goat anti-rabbit immunoglobulin (Code A 8025, Sigma) diluted 1/2000 in TBS/Tw containing 0·5 M NaCl. The membranes were washed and developed with nitroblue tetrazolium and potassium 5-bromo-4-chloro-3-indolylphosphate.

Clinical material

The animals included in this study originated from a breeding experiment, involving multiple ovulations and embryo transfers to propagate new generations. Feeding and rearing of the animals have previously been reported.[30]

Five hundred and sixty-nine calves were analysed (260 heifers and 309 bull-calves) from four dairy breeds, Red Danes, Holstein, Jersey, and Red-Pied. The animals were housed at two different breeding stations: Egtved Avlsstation (bull-calves) and Ammitsbøl Skovgaard (heifers). Blood samples were drawn from an intravenous catheter into heparin at a fixed time of day (08:00) after overnight fasting. The plasma was immediately separated and stored at −20°.

Recording of infections

The calves were monitored for pulmonary infections from day 42 to day 336 after birth. Only infections considered serious enough to justify antibiotic treatment were recorded. Recurrence of infection within 7 days of first recording was considered as belonging to the original case, and was not counted as a new infection. The diagnosis and recording of diseases were made by experienced veterinary practitioners, who did not know the conglutinin levels in the individual animals. The calves were grouped in batches of 5–12 animals at about 9 months of age (242–317 days, mean 270 days).

Genetic analysis

Conglutinin concentrations were $\log_e$ transformed in order to obtain an approximately normal distribution. Values below the detection limit of the assay (1·25 μg/ml) were coded as 0·00 (zero) on the $\log_e$ scale for the statistical analysis.

Number of infections was $1+\log_e$-transformed in order to obtain an approximately normal distribution for the genetic analysis. Animals without infection were assigned a value of 0·00. Mean values in the measured scale were obtained by back-transformation.

Variation between animals with regard to conglutinin concentration and in disease frequency was analysed for effects of genetic background, other systematic factors and random variation from and within the testing environment. The genetic constribution to the variation between animals was expressed as the heritability (h^2), giving the ratio between the genetic variation and the total phenotypic variation. Random variation between batches included seasonal variation and environmental factors common to animals tested on the same day. These factors are believed to include exposure to fluctuating infections occurring in the barn in the same time period. The relative importance of the batch–environment effect (c^2) was calculated as the ratio of the batch variance to the total phenotypic variance. The phenotypic variance consisted of genetic, environmental and residual variance. Variance attributable to systematic factors, such as breed of animal and testing station was excluded from the phenotypic variance estimate. Similarly, components of covariance between traits were used

Table 1. Genealogical tree of the probands included in this study

Probands	Parents	Grandparents	Great-grandparents
		38 paternal grandsires	28 great-grandsires
	56 sires		
		56 paternal granddams	44 great-grandsires
309 males			
260 females			
		74 maternal grandsires	51 great-grandsires
	177 dams		
		153 maternal granddams	92 great-grandsires

Female great-grandparents are not assumed to provide relationship information over and above that already accounted for by their offspring.

for calculation of genetic correlations (r_A), batch correlations (r_C), residual correlations (r_e), and phenotypic correlations (r_P).

The relationship between calves was of a complex nature consisting of full and half sibs, dam offspring, and through common grandparents, as indicated by the genealogical tree shown in Table 1.

At least three generations of ancestors were known for each tested individual. Because male and female calves were housed at separate testing stations, conglutinin concentrations and disease records were initially considered as different traits, linked through genetic correlation in bivariate analysis of the traits. A multivariate analysis of variance was performed using the individual animal model where full relationship was used to estimate the genetic component of variance and co-variance. The model included fixed factors for breed, testing station/sex and year of birth. Test batch was included as a random factor. The effects of all factors and the variance components were simultaneously estimated through restricted maximum likelihood methods.[31] The effect of fixed factors was tested using the *F*-test.

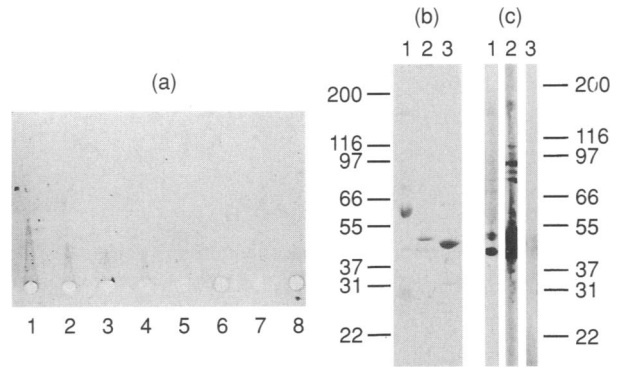

Figure 1. Characterization of the rabbit anti-bovine conglutinin antiserum. (a) rocket immunoelectrophoresis of purified conglutinin (wells 1–4: 20–2·5 μg/ml) and purified CL-43 (wells 5–8: 20–2·5 μg/ml). (b) SDS–PAGE of: (1) ≈5 μg reduced and carboxyamidated bovine serum proteins precipitated by 5% (w/v) PEG 6000; (2) 5 μg reduced and carboxyamidated purified conglutinin; (3) 5 μg partly purified CL-43 (lane 3). (c) Same samples as in (b) analysed by SDS–PAGE followed by Western blotting and developing with the anti-bovine conglutinin antiserum.

RESULTS

Characterization of the anti-conglutinin antiserum

The specificity of the rabbit anti-conglutinin antiserum was analysed by rocket immunoelectrophoresis and Western blots. Figure 1a shows the specificity of the anti-conglutinin antibody analysed by rocket immunoelectrophoresis. Purified conglutinin gave quantitative precipitation arcs, while the homologous serum collectin CL-43 gave no precipitations. Figure 1b shows SDS–PAGE of bovine serum proteins precipitated by 5% (w/v) polyethylene glycol (PEG) 6000 (lane 1), purified conglutinin (lane 2) and purified CL-43 with a minor MBL contamination (lane 3), all reduced and carboxyamidated and stained by Commassie Brilliant Blue. A parallel run of the same material was blotted onto a PVDF membrane and developed with the rabbit anti-conglutinin antiserum (Fig. 1c). Two bands corresponding to native and truncated conglutinin were stained in the 5% PEG 6000 precipitate of bovine serum (lane 1), and the same bands were stained in the purified preparation of conglutinin (lane 2), while no staining was seen corresponding to purified CL-43 (lane 3), or to MBL which can be seen as a minor contamination of the CL-43 preparation in Fig. 1b, lane 3, at about 32 000 MW.

Concentrations of conglutinin in plasma

The concentration of conglutinin in the 569 samples tested is shown in Fig. 2 and geometric mean values for breeds and gender in Table 2. The geometric mean concentration in males (15·5 μg/ml) was significantly higher than that in females (8·1 μg/ml) (*F*-test, $P<0{\cdot}001$). Twelve animals (10 females and 2 males) had conglutinin levels below the detection level of the assay. Significant differences were found between different dairy breeds. The genetic influence on the concentration of conglutinin was strong in both environments/sexes, as shown by the high heritability (female: $h^2=0{\cdot}45\pm0{\cdot}11$; male: $h^2=0{\cdot}67\pm0{\cdot}11$) for both sexes (Table 3). The ranking of breeds was the same in both sexes. This was in agreement with a genetic correlation between the sexes approaching unity, showing that conglutinin is genetically the same trait in male and female calves, although the mean level differ between the sexes. This finding further justifies a pooled heritability estimate comprising animals of both sexes, after including testing station as a fixed effect in the model.

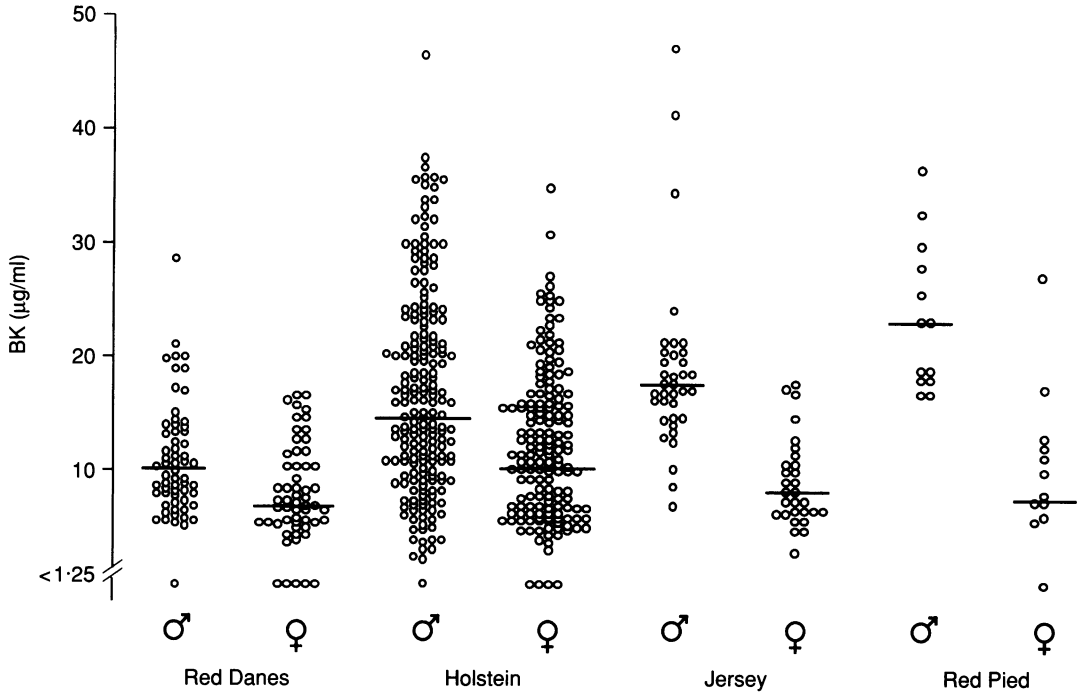

Figure 2. Conglutinin concentrations in 569 calves. The animals were of four different breeds; males and females are shown separately. (1) Red Danes, males ($n=61$), females ($n=57$); (2) Holstein, males ($n=199$), females ($n=162$); (3) Jersey, males ($n=36$), females ($n=29$); (4) Red-Pied, males ($n=13$) females ($n=12$). Geometric mean concentrations are indicated. BK: conglutinin.

Table 2. Conglutinin concentration and number of infections in male and female calves of four dairy breeds

			Conglutinin		Infection	
Sex	Breed	*n*	log unit	GM (μg/ml)	1+log unit	GM (no. of infections)
Males	Red Danes	61	2·30±0·11	10·0	1·69±0·13	2·0
	Holstein	199	2·67±0·08	14·5	1·85±0·09	2·3
	Jersey	36	2·86±0·13	17·5	1·66±0·16	1·9
	Red-Pied	13	3·13±0·22	22·9	1·88±0·26	2·4
Males	Pooled	309	2·74±0·79	15·5	1·77±0·10	2·2
Female	Red Danes	57	1·89±0·12	6·7	1·24±0·14	1·3
	Holstein	162	2·31±0·08	10·1	1·33±0·10	1·4
	Jersey	29	2·12±0·15	8·3	1·18±0·16	1·2
	Red-Pied	12	2·02±0·22	7·5	1·10±0·26	1·1
Female	Pooled	260	2·09±0·81	8·1	1·20±0·10	1·2

Results are given as means in $\log_e$ units (±SEM) and corresponding geometric means (GM) in μg/ml and number of infections

The batch variability of conglutinin concentration was small in both sexes–environments (heifers, $c^2=0{\cdot}08\pm0{\cdot}12$; bulls, $c^2=0{\cdot}04\pm0{\cdot}15$).

Infection of calves

The frequency of respiratory diseases varied between 0 and 13 per calf, and was twofold higher for male (geometric mean 2·2 infections) than for female calves (geometric mean 1·2 infections) (Table 2). Breed effects were pronounced. Number of respiratory diseases had a reasonably high heritability in both environments/sexes and the genetic correlation between male and female approached unity (Table 3), which justified a pooled analysis leading to an overall heritability estimate of $h^2=0{\cdot}31\pm0{\cdot}07$.

The batch variation, including seasonal variation, in relation to the number of infections was larger than the corresponding batch variability in conglutinin concentrations, but smaller than the heritability estimates. A pooled estimated of $c^2=0{\cdot}13\pm0{\cdot}08$) was obtained.

Table 3. Genetic parameters for conglutinin and observed frequecy of respiratory infections in calves of four dairy breeds

	Conglutinin		Infections		Conglutinin-infections	
Parameter/sex	h^2	c^2	h^2	c^2	r_a	r_p
Males	0·67±0·11	0·04±0·15	0·26±0·09	0·09±0·10	−0·50±0·18	−0·18
Females	0·45±0·11	0·08±0·12	0·49±0·12	0·18±0·14	−0·55±0·18	−0·11
Pooled	0·52±0·07	0·07±0·09	0·31±0·07	0·13±0·08	−0·52±0·13	−0·12
r_a: Males–females	1·00±0·11		0·91±0·20			

Results are heritability (h^2±SE) and batch variability coefficients (c^2±SE), and genetic (r_a±SE) and phenotypic (r_p) correlations.

Conglutinin–infection relationships

A clear negative genetic correlation between plasma conglutinin and number of recorded diseases was revealed in the bivariate genetic analysis (Table 3). This was found independently in both females and males to be of similar size, giving a pooled estimate of $r_a = -0{\cdot}52 \pm 0{\cdot}13$). Furthermore, the phenotypic relationship was also negative ($r_P = -0{\cdot}12$), although of smaller magnitude than the genetic correlation. The disease frequency in the group of calves with undetectable low plasma conglutinin was not higher than in the group with detectable levels of conglutinin.

DISCUSSION

We have applied a quantitative conglutinin assay to plasma samples from 569 calves with complex pedigree relationships, from four different breeds. A significant difference in the geometric means of conglutinin concentrations was found between bull-calves and heifers in all four breeds, bull-calves having higher concentrations of conglutinin than heifers. Bull-calves and heifers were stabled at different stations and the feeding regime for the heifers included a diet of lower energy content than that fed to the bull-calves.[30] It can therefore not be excluded that factors from the outer environment could explain some of the difference in conglutinin concentration between the two sexes. However, to our knowledge there are no reports in the literature suggesting that different, but adequate, feeding and stabling conditions would have a significant influence on the concentration of a serum protein.

The frequency of recorded diseases was high in both herds. This may be a result of the experimental station continuously receiving young calves from large number of commercial herds. Also, because the animals were of high breeding value the attention level was high and the treatment threshold likely lower than in commercial herds. The bull-calves suffered a higher number of infections, and it cannot be excluded that this may have influenced the general conglutinin level. Calves of both sexes raised under the same conditions must be tested for conglutinin to answer these questions.

Significant differences in concentrations of conglutinin were also found between the breeds. Parallel findings have been reported in man, where higher concentrations of MBL are found in Eskimos than in Caucasians and Black Africans.[32]

Several reports indicate that low levels of MBL correlate with recurrent infections in man. MBL deficiency is linked to three different mutations in the collagen region of the molecule, all leading to disruption of Gly–Xaa–Yaa triplets that would impair assembly of the intact functional molecule.[7,26] Recently, mutations in the 5'-flanking regulatory region have also been described and found to be associated with decreased levels of MBL.[33]

MBL concentrations in man are highly variable (from below 10 ng/ml to 10 μg/ml). Increased susceptibility to infection is observed only in persons homozygous for the mutations, which result in levels of MBL below 10 ng/ml.[25] In the present study the variation of the conglutinin level is also wide (from below 1·25–47 μg/ml), and a polygenic inheritance of conglutinin concentrations is clearly established by the genetic analysis. We also show that low levels of conglutinin are significantly associated with an increased susceptibility to respiratory infection. 12 of 569 calves had levels of conglutinin below the detection limit of the assay. If these animals are assumed to be homozygous for a single recessive allele causing low concentrations, the frequency of this allele can be estimated as $q = 0{\cdot}15$. Obviously further investigations are required to establish the number of alleles involved. The frequency estimated from the assumption of regulation by a single recessive allele is comparable to the gene frequencies for the different alleles leading to the MBL deficiency in man. These vary from $q = 0{\cdot}02$–$0{\cdot}23$, depending on the allele and the race. It is thus possible that a single mutation in the collagen region of the conglutinin gene, leading to a disruption of the collagen triplet, is responsible for the low levels of conglutinin. Different alleles of conglutinin have already been found, but all mutations described so far have been either silent or located at positions where the exchange of a single amino acid residue would be expected to have little functional or structural effect.[34]

This study is the first demonstration of a genetic association between the level of conglutinin and the frequency of respiratory infection in cattle. The relationship was of similar significance in both sexes, and in two different environments. The conglutinin level was clearly associated with disease frequency. These findings suggests that selection against respiratory disease is possible in animal husbandry and in achieving this goal measurement of conglutinin in plasma may be a helpful parameter.

The group of calves used in this study were only observed for infection from the 42nd day of life. It is well known that ≈5% of calves die from infection during the first weeks of life, and one may speculate that deficiency of conglutinin (or other collectins) may have an even more pronounced effect on health in these calves than in older animals.

The high heritability of conglutinin concentrations suggests that conglutinin may be an independent parameter of importance in breeding programmes directed at improving the health of cattle. The fact that respiratory infection on its own has a

genetic association and that this trait is not fully accounted for by an association with plasma conglutinin concentrations also highlights the importance of extending this type of investigation to encompass the two other serum collectins, MBL and CL-43.

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